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Identification Of Paraproteins Via Serum Immunofixation or Serum Immunosubtraction and Immunoturbidimetric Quantitation of Serum Immunoglobulins in the Laboratory Testing for Monoclonal Gammopathies

A Comparison of Methods

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• ***Context.***—**In laboratory testing for monoclonal gammopathies, paraproteins are identified via serum immunofixation or serum immunosubtraction and immunoturbidimetric quantitation of serum immunoglobulins is often used.**

Objective.—**To evaluate methodic differences between serum immunofixation and serum immunosubtraction as well as in the quantitation of serum immunoglobulins on different clinical chemical platforms.**

Design.—**Three hundred twenty-two unique routine patient samples were blinded and used for comparison between serum immunofixation on Sebia's HYDRASIS 2 and serum immunosubtraction on Sebia's CAPILLARYS 2 as well as between quantitation results of immunoglobulin A, G, and M on Abbott's ARCHITECT c16000PLUS and Roche's Cobas c 502 module. Microsoft Excel 2019 with the add-on Abacus 2.0 and MedCalc were used for statistical analysis and graphic depiction via bubble diagram, Passing-Bablok regressions, and Bland-Altman plots.**

Results.—**The median age of patients was 75 years and samples with paraproteinemia were nearly evenly split between sexes. Paraprotein identification differed remarkably between immunofixation and immunosubtraction. Quantitation of serum immunoglobulins showed higher values on Abbott's ARCHITECT c16000PLUS when compared with Roche's Cobas c 502 module.**

Conclusions.—**Identification of paraproteins via serum immunosubtraction is inferior to serum immunofixation, which can have implications on the diagnosis and monitoring of patients with monoclonal gammopathy. If immunoturbidimetric quantitation of immunoglobulins is used for follow-up, the same clinical-chemical platform should be used consistently.**

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Monoclonal gammopathies are plasma cell disorders associated with the production of monoclonal immunoglobulins (Ig), also known as M-protein.[1–5] They range from usually asymptomatic monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma to solitary plasmocytoma, monoclonal gammopathy of renal significance, malignant plasma cell disorders and lymphoid disorders, including multiple myeloma (MM), primary amyloidosis, and Waldenström macroglobulinemia.[1–9]

Almost all cases of MM begin as an MGUS with an M-protein that may be present in serum as early as a decade before diagnosis of MM.[10] While MM comprises approximately 10% of hematologic malignancies, MGUS is vastly more prevalent and progressively increasing with age such that by age 50 it is found in 1:100 individuals and by age 70 in 1:20.[11] MM usually occurs in older people but it has been reported that 2% of MM cases can occur in individuals younger than 40 years of age, which means that MGUS was likely present as early as in their 20s.[12]

In monoclonal gammopathies, the full laboratory workup for initial diagnosis and disease relapse includes a complete blood count and differential, a peripheral blood smear, a chemistry screen including calcium and creatinine, serum protein electrophoresis, serum immunofixation (IFE), immunoturbidimetric or immunonephelometric quantification of serum Ig, routine urinalysis, 24-hour urine collection for electrophoresis and immunofixation, serum β_2-microglobulin, lactate dehydrogenase, and measurement of serum free light chains.[2,4,6,9] Flow cytometry and molecular characterization techniques can supplement this workup.[5,13–16]

Characterization of the paraprotein is especially important as it is needed for prognosis and follow-up after therapy.[9] For this, immunofixation is the gold standard. Immunosub-

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traction (ISE) may be used in its place but is supposed to be less sensitive.[2,6]

Apart from paraprotein identification and electrophoretic position, key aspects of prognosis and follow-up of monoclonal gammopathies rely upon obtaining a reproducible, accurate quantification of M-proteins via measurement of the size of the M-spike itself by serum and/or urine electrophoresis or immunochemical measurement of the involved isotype in serum samples.[9]

The immunoturbidimetric or immunonephelometric quantitation of Ig are simple automated methods that measure both monoclonal and polyclonal Ig. Therefore, monoclonal protein concentration may be significantly overestimated, especially with pentameric IgM paraproteins and in nephelometry.[2,4,17,18] Nonetheless, nephelometric or turbidimetric quantitation of IgA, IgG, and IgM can be used to monitor hematologic disease when there is little polyclonal Ig synthesis and should be used when IgA or IgM M-protein migrates in the β fraction or the electrophoretic IgG M-spike is more than 30 g/L.[4]

In comparison to immunoturbidimetric and immunonephelometric quantitation, estimation of monoclonal protein via serum protein (capillary zone) electrophoresis can suffer from a lack of comparability due to the subjective nature of M-spike demarcation.[9] Interlaboratory comparability might be further compromised due to nonuniform use of perpendicular drop and tangent skimming for electrophoretic M-spike quantitation. Conventional serum protein electrophoresis with protein dye gel staining for determination of M-protein concentration can also suffer from a dye-saturation effect of the gel, especially in cases of high M-protein concentrations.[9,18]

For the reasons stated above results of immunometric quantitation and protein electrophoresis do not always agree and for patients with measurable monoclonal protein in serum, both electrophoretic studies and quantitative Ig measurements are recommended to assess response to therapy.[2] When this is done, the same methods (and ideally the same laboratory) should be continuously used for follow-up.[2,18]

In addition to aforementioned differences between electrophoretic studies and immunometric Ig quantitation, there can be methodic differences in the immunoturbidimetric quantitation of IgA, IgG, and IgM on different analytical platforms.

The aim of this study was to evaluate methodic differences between serum immunofixation on HYDRASIS 2 and serum immunosubtraction on CAPILLARYS 2 (Sebia SA) as well as in the turbidimetric quantitation of IgA, IgG, and IgM on ARCHITECT c16000PLUS (Abbott Laboratories) and Cobas c 502 module (Roche Diagnostics).

METHODS

From April 9, 2018 to May 9, 2018, aliquots were taken from all routine blood samples with requested serum capillary zone electrophoresis, serum free light chains and ISE.

The total number of unique patient samples was 360. Thirty-eight of these samples had to be excluded due to insufficient volume of leftover serum.

Aliquots of the remaining 322 blood samples were blinded and used for comparative paraprotein identification on Sebia's HYDRASIS 2 and CAPILLARYS 2 (Sebia Labordiagnostische Systeme GmbH, Fulda, Germany) and comparative Ig measurements on Abbott's ARCHITECT c16000PLUS (Abbott Medical GmbH, Wetzlar, Germany) as well as Roche's Cobas c 502 module (Roche Deutschland Holding GmbH, Mannheim, Germany) according to the manufacturers' specifications. On both clinical chemical platforms, the following reference intervals for Ig were used in accordance with the data published by Dati et al[19]: IgA: 70 to 400 mg/dL, IgG: 700 to 1600 mg/dL, IgM: 40 to 230 mg/dL.

If measurements could not be done on the same day, the aliquots were stored at 4°C and evaluated within the next 3 days. IFE and ISE were evaluated independently of Ig quantitation, additional measurements and each other (ie, at the time of evaluation via ISE the results of IFE and quantitative Ig measurements were unknown to the reviewer and vice versa). All evaluations were done by the same reviewer, who was familiar with both IFE and ISE.

General study population statistics were assessed via Microsoft Excel 2019 (Microsoft Corporation, Redmond, Washington).

Results of paraprotein identification were compared using a bubble diagram. Agreement between IFE and ISE was assessed via contingency table and Kendall's Tau (τ), a coefficient with a range of $-1 \leq \tau \leq 1$. Perfect agreement would result in a coefficient of 1, perfect disagreement in a coefficient of –1.[20]

Results of the immunoturbidimetric Ig assays were compared using Passing–Bablok regression analysis.

The Spearman rank correlation coefficient (r_S) was determined to evaluate correlation between immunoturbidimetric methods. The correlation was graded as proposed by Evans with less than 0.20 indicating a very weak, 0.20 to 0.39 a weak, 0.40 to 0.59 a moderate, 0.60 to 0.79 a strong, and more than 0.80 a very strong correlation.[21]

Agreement of immunoturbidimetric Ig assays was compared using Bland–Altman plots. Concordance was calculated using contingency tables. Cohen κ coefficients were calculated for interobserver agreement. The results of Cohen κ were evaluated according to Altman as follows: less than 0.2 indicates a poor, 0.21 to 0.40 a fair, 0.41 to 0.60 a moderate, 0.61 to 0.80 a good, and more than 0.81 a very good concordance.[22]

MedCalc (Version 18.3; MedCalc Software, Ostend, Belgium) and Microsoft Excel 2019 with the add-on Abacus 2.0 (Version 1.40.36.02; LABanalytics GmbH, Germany) were used for statistical analyses and graphical depictions. A threshold of $P < .05$ was set for statistical significance.

RESULTS

General Study Population Statistics

Aliquots of 322 unique serum samples were included in the comparison study. The samples were nearly evenly spread across male and female patients. The age span covered 20 to 97 years and the median age was 75. The patients' characteristics as well as the total number of identified paraproteins per age group and sex can be found in Table 1.

Paraprotein Identification

Paraprotein identification using Sebia's HYDRASIS 2 took a total of 3454 minutes. The processing time on Sebia's CAPILLARYS 2 was 2732 minutes, equaling a delta of 722 minutes or 12.03 hours across the 322 evaluated samples. All in all, 17 different paraproteins were identified on the 2 platforms. An overview of the identified paraproteins and their respective identification numbers is shown in Table 2. Figure 1 shows the bubble diagram after statistical evaluation in a contingency table. τ was .677 ($P < .001$), indicating good agreement between serum immunofixation and immunosubtraction.

Quantitative Immunoglobulins

IgA.—Passing-Bablok linear regression and the r_S of .987 ($P < .001$) showed a very strong correlation between measured IgA results on Abbott's ARCHITECT 16000Plus

Table 1. General Study Population Statistics			
Characteristics (n = 322)	**n (%)**	**n (%) Positive Immuno-Fixations (n = 69)**	**n (%) Positive Immuno-Subtractions (n = 51)**
Sex			
Female	159 (49)	33 (48)	24 (47)
Male	163 (51)	36 (52)	27 (53)
Age, yr	20–97		
Median	75		
<40	16 (5)	0 (0)	0 (0)
40–49	16 (5)	1 (1)	1 (2)
50–59	41 (13)	6 (9)	2 (4)
60–69	62 (19)	15 (22)	11 (22)
70–79	92 (29)	25 (36)	23 (45)
≥80	95 (30)	22 (32)	14 (28)

and Roche's cobas 502. The Bland-Altman plot revealed a mean positive bias of 4.5% when measurements on Abbott's ARCHITECT 16000PLUS were compared to values on Roche's Cobas 502 module. Evaluation of interobserver agreement via contingency analysis resulted in a Cohen κ of 0.63, which equals good agreement (Table 3). Figure 2, A and B depicts the Passing-Bablok linear regression and the Bland-Altman plot for IgA. Three samples were excluded from statistical analyses because their IgA was below the lower limit of quantitation on Abbott's ARCHITECT 16000PLUS and 1 sample was excluded, because measured IgA was below the lower limit of quantitation on Roche's Cobas 502 module.

IgG.—Passing-Bablok linear regression and the r_S of .969 ($P < .001$) showed a very strong correlation between measured IgG results on Abbott's ARCHITECT 16000Plus and Roche's Cobas 502. The Bland-Altman plot revealed a mean positive bias of 13.7% when measurements on Abbott's ARCHITECT 16000PLUS were compared with values on Roche's Cobas 502 module. Evaluation of interobserver agreement via contingency analysis showed a Cohen κ of 0.21, which equals fair agreement (Table 3). Figure 2, C and D depicts the Passing-Bablok linear regression and the Bland-Altman plot for IgG. Three samples were excluded from statistical analyses because their IgG was below the lower limit of quantitation on Abbott's ARCHITECT 16000PLUS and 1 sample was excluded, because the value was above the upper limit of quantitation on Abbott's ARCHITECT 16000PLUS.

IgM.—Passing-Bablok linear regression and the r_S of .979 ($P < .001$) showed a very strong correlation between measured IgM results on Abbott's ARCHITECT 16000Plus and Roche's Cobas 502. The Bland-Altman plot revealed a mean positive bias of 5.7% when measurements on Abbott's ARCHITECT 16000PLUS were compared with values on Roche's Cobas 502 module. Evaluation of interobserver agreement via contingency analysis showed a Cohen κ of 0.63, which equals good agreement (Table 3). Figure 2, E and F depicts the Passing-Bablok linear regression and the Bland-Altman plot for IgM. Nineteen samples were excluded from statistical analyses because their IgM was

Table 2. Distribution of Identified Paraproteins			
Diagnosis IDs	**Paraprotein**	**Serum Immunofixation**	**Serum Immunosubtraction**
0	None	253	271
1	IgA-heavy chain	1	0
2	IgA-κ	7	2
3	IgA-λ	3	1
4	IgG-κ	12	18
5	IgG-κ & IgG-λ	1	2
6	IgG-κ & IgM-κ	1	0
7	IgG-λ	27	20
8	IgG-λ & IgG-λ	1	0
9	IgG-λ & IgM-κ	1	0
10	IgG-λ & IgM-λ	1	0
11	IgM-κ	7	6
12	IgM-κ & IgA-κ	0	1
13	IgM-κ & IgG-λ	1	0
14	IgM-κ & IgM- λ	1	0
15	IgM-λ	3	0
16	κ-light chain	1	1
17	κ- & λ-light chains	1	0

Abbreviation: IDs, identification numbers.

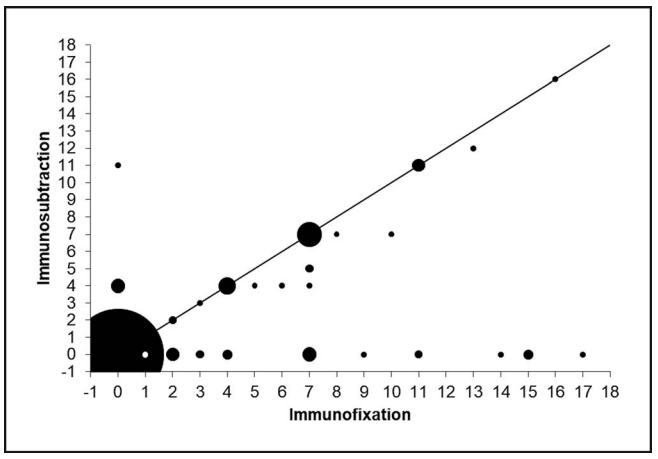

Figure 1. *Bubble diagram of identified paraproteins. The bubble size is equivalent to the number of patient samples with the respective paraprotein. If serum immunofixation (IFE) and serum immunosubtraction (ISE) showed complete agreement, all bubbles would be placed on the reference line. The numbers on the x-axes and y-axes correspond to the identification numbers and associated paraproteins in Table 2 (n = 322).*

below the lower limit of quantitation on Abbott's ARCHITECT 16000PLUS.

DISCUSSION

As demonstrated by our results, IFE and ISE still produce markedly different results after more than 40 years since the first description of ISE by Aguzzi and Poggi[23] in 1977. In our study, IFE detected and identified a total of 69 paraproteinemias, while ISE only detected monoclonal proteins in 51 samples, a difference of 26%. ISE failed to detect 6 of 7 samples with biclonal paraproteinemias as well as 8 of 11 monoclonal paraproteins involving IgA and 4 of 10 monoclonal paraproteins involving IgM identified by IFE. For monoclonal paraproteins involving IgG, the total number of detections was 39 in IFE and 38 in ISE.

This is in accordance with several other publications comparing IFE to ISE on different platforms, including one that compared Sebia's CAPILLARYS 2 with Sebia's HYDRASIS[24–27]: In the study of Bossuyt et al[24] 5 of 58 paraproteinemias (ie, 9%) were missed or not identified by ISE when compared with IFE.

When Henskens et al[25] compared 74 serum samples containing monoclonal proteins, IFE detected and identified 73 while ISE identified only 69, a difference of ca. 6%.

In the study of Litwin et al[26] ISE was evaluated by 4 independent reviewers. Forty-eight samples with paraprotein were identified via IFE. However, the reviewers could only identify between 29 and 36 monoclonal proteins via ISE, a difference of 25% to 40%. All of the reviewers missed the biclonal paraprotein in this study and scored particularly poorly in the ability to correctly immunotype monoclonal proteins of the IgM isotype, with only 30% to 40% correctly identified. In addition, polyclonal or normal patterns were partially misinterpreted as IgG monoclonal proteins.[26] A similar effect could be observed for IgG-κ M-protein in our study, ISE identified monoclonal IgG-κ in 18 samples, while IFE characterized 6 of those as normal patterns. On the other hand, ISE also under identified monoclonal IgG-λ in our study, detecting only 20 of 27 samples identified by IFE. Litwin et al[26] and Bossuyt et al[24] apparently did not make a similar observation for monoclonal IgG-λ. However, their ISE were performed with a Paragon CZE 2000 system (Beckman Instruments, Brea, California) and other antisera than ours, which might be an explanation for this.

Table 3. Concordance of Immunoglobulin Quantitation

	Roche	Abbott
Immunoglobulin A		
Decreased	14	14
Normal	267	259
Elevated	41	49
Cohen κ: 0.63		
Immunoglobulin G		
Decreased	47	29
Normal	248	251
Elevated	27	42
Cohen κ: 0.21		
Immunoglobulin M		
Decreased	50	45
Normal	256	256
Elevated	16	21
Cohen κ: 0.63		

Yang et al[27] found that ISE produced 7 discrepant results, when compared with 43 monoclonal proteins identified via IFE, a difference of 16%. In 6 cases paraprotein involving IgM was not detected by immunosubtraction. Three of them were biclonal multiple myelomas. ISE also missed the free κ light chains identified via IFE in 1 case of multiple myeloma. Assessment was done by 8 individual reviewers in this study.

All in all, ISE particularly missed samples containing paraprotein involving IgM, IgA, and free light chains as demonstrated by our study and several other authors. This discrepancy might be explained by the failure to detect "hidden paraproteins" via ISE. These paraproteins cannot be detected by capillary zone electrophoresis but can be revealed by IFE, often due to the M-protein being located within the peaks of the α-2- or β-fractions.[24,27,28] In addition, monoclonal proteins missed by ISE tended to be of low concentration.[3,26,27] M-proteins that are barely or not detectable via capillary zone electrophoresis and ISE but can be detected via IFE are synonymous with IFE MGUS.[29] Up to 70% of these paraproteinemias may persist and require continuous monitoring, while as much as 3.2% of cases can progress to plasma cell malignancy within 4 years of first detection.[29]

Because patients benefit from early detection of monoclonal gammopathies and incorrect classification or missed identification of M-proteins can adversely affect the patients' diagnosis, prognosis and treatment, the method with the greatest possible sensitivity (ie, IFE) should be used whenever possible.[24,30–32] Therefore, ISE was discontinued in our laboratory.

While apparently inferior to IFE with regard to M-protein detection and identification, ISE has several advantages:

Owing to its greater automation and decreased hands-on time ISE can result in financial and labor savings as well as improved turnaround times, when compared with IFE.[24–26] ISE might be performed at any time of the day with much less effort than IFE and without the need for batching 4 or 9 samples per individual run. In our study ISE saved 722

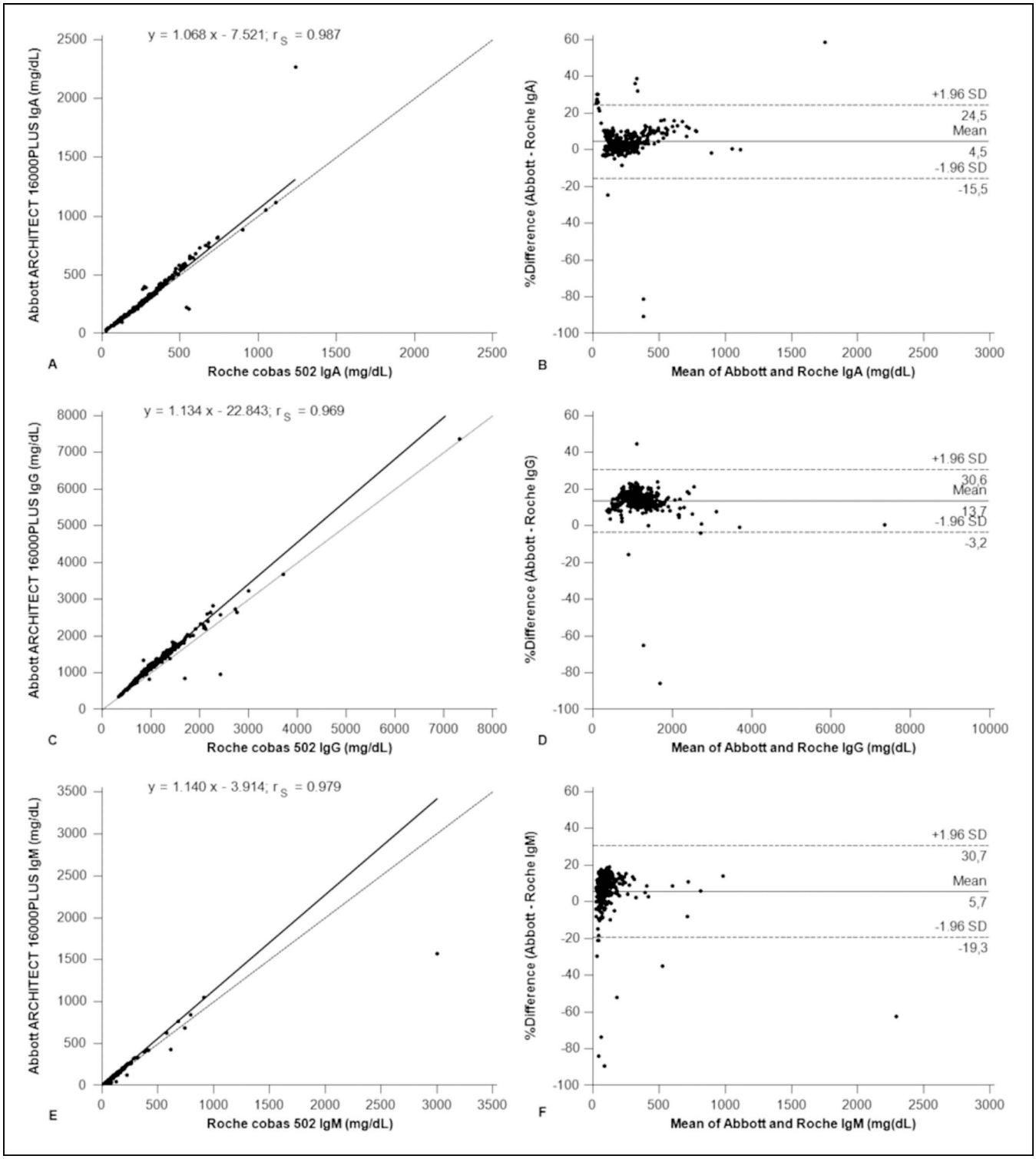

Figure 2. *Comparison of immunoglobulin quantitation on Roche's Cobas 502 module and Abbott's ARCHITECT 16000PLUS analyzer. Passing-Bablok linear regressions and Bland-Altmann plots were performed for immunoglobulin (Ig) A (A and B), IgG (C and D), and IgM (E and F). All evaluated Ig showed a very strong correlation when measurements on Abbott's ARCHITECT 16000PLUS and on Roche's Cobas 502 module were compared. Results on Abbott's ARCHITECT showed a positive bias when compared to Roche's Cobas 502 module, indicating generally higher values of IgA, IgG, and IgM on that clinical chemical platform. n (IgA) = 318; n (IgG) = 318; n (IgM) = 303.*

minutes when compared with IFE (ie, 2 minutes and 15 seconds per sample). This might be beneficial in smaller or hospital laboratories, where less personnel are on hand and results might be expected as quickly as possible.

Greater automation including barcode readers integrated in ISE machines also decreases the risk for human error when compared with IFE, because no manual dilution steps, sample application and film labelling are necessary.

However, owing to the fact that ISE can miss or misidentify monoclonal and biclonal paraproteinemias and ISE patterns were found to be difficult to interpret with confidence as demonstrated by our findings and the cited studies, results should be confirmed by IFE, if ISE is employed.[24–27]

With regard to the quantitation of Ig, we found that measurements on Abbott's ARCHITECT 16000Plus tended to be higher, when compared with results on Roche's Cobas 502. This positive bias was highest for IgG and lowest for IgA and statistically significant for every evaluated Ig. Owing to this, the reference ranges published by Dati et al[19] might not be suitable for measurements on Abbott's ARCHITECT 16000Plus and the higher reference ranges published by the manufacturer or individually established reference ranges should be used.

There was only good agreement between the methods for IgA and IgM and agreement for IgG was fair. Comparability of Ig quantitation appears to be limited between platforms.

Therefore, when and if Ig quantitation is used for monitoring of monoclonal gammopathies, the same method and the same laboratory should be used consistently. If that is not possible, the possibility of bias between methods as well as analytical platforms must be kept in mind and the limited comparability must be considered during patient follow-up.

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